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CANCER CHEMOPROTECTIVE FOOD PRODUCTS

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BACKGROUND OF THE INVENTION

I. Field of Invention

This invention relates to a dietary approach to reducing the level of carcinogens in animals and their cells and thereby reducing the risk of developing cancer. In particular, this invention relates to the production and consumption of foods which are rich in cancer chemoprotective compounds. More specifically, this invention relates to chemoprotective compounds that modulate mammalian enzymes which are involved in metabolism of carcinogens. This invention relates to food sources which are extremely rich in compounds that induce the activity of Phase 2 enzymes, without inducing biologically significant activities of those Phase 1 enzymes that activate carcinogens.

II. Background

It is widely recognized that diet plays a large role in controlling the risk of developing cancers and that increased consumption of fruits and vegetables reduces cancer incidence in humans. It is believed that a major mechanism of protection depends on the presence of chemical components in plants that, when delivered to

mammalian cells, elevate levels of Phase 2 enzymes that detoxify carcinogens.

Early studies on the mechanism of chemoprotection by certain chemicals assumed that these chemoprotectors induced activities of monooxygenases, also known as Phase 1 enzymes or cytochromes P-450. However, Talalay *et al.*, [reviewed in "Chemical Protection Against Cancer by Induction of Electrophile Detoxication (Phase II) Enzymes" In: CELLULAR AND MOLECULAR TARGETS OF CHEMOPREVENTION, L. Wattenberg *et al.*, CRC Press, Boca Raton, FL, pp 469-478 (1992)] determined that administration of the known chemoprotector butylated hydoxyanisole (BHA) to rodents resulted in little change in cytochromes P-450 (Phase 1 enzyme) activities, but profoundly elevated Phase 2 enzymes. Phase 2 enzymes such as glutathione transferases, NAD(P)H:quinone reductase (QR) and glucuronosyltransferases, detoxify DNA-damaging electrophilic forms of ultimate carcinogens. Selective inducers of Phase 2 enzymes are designated monofunctional inducers. Prochaska & Talalay, *Cancer Res.* 48: 4776-4782 (1988). The monofunctional inducers are nearly all electrophiles and belong to 8 distinct chemical classes including (1) diphenols, phenylenediamines and quinones; (2) Michael reaction acceptors containing olefins or acetylenes conjugated to electron-withdrawing groups; (3) isothiocyanates; (4) 1,2-dithiole-3-thiones; (5) hydroperoxides; (6) trivalent inorganic and organic arsenic derivatives; (7) heavy metals with potencies related to their affinities for thiol groups including Hg^{2+}, and Cd^{2+}; and (8) vicinal dimercaptans. Prestera *et al.*, *Proc. Natl. Acad. Sci. USA* 90: 2963-2969 (1993). The only apparent common property shared by all of these inducers is their ability to react with thiol groups.

Chemoprotective agents can be used to reduce the susceptibility of mammals to the toxic and neoplastic effects of carcinogens. These chemoprotectors can be of

plant origin or synthetic compounds. Synthetic analogs of naturally occurring inducers have also been generated and shown to block chemical carcinogenesis in animals. Posner et *al.*, *J. Med. Chem.* <u>37</u>: 170-176 (1994); Zhang et *al.*, *Proc. Natl. Acad. Sci. USA* <u>91</u>: 3147-3150 (1994); Zhang et *al.*, *Cancer Res. (Suppl)* <u>54</u>: 1976s-1981s (1994).

Highly efficient methods have been developed for measuring the potency of plant extracts to increase or induce the activities of Phase 2 enzymes. Prochaska & Santamaria, *Anal. Biochem.* <u>169</u>: 328-336 (1988) and Prochaska *et al.*, *Proc. Natl. Acad. Sci. USA* <u>89</u>: 2394-2398 (1992). In addition, these methods have been employed for isolating the compounds responsible for the inducer activities in plants and for evaluating the anticarcinogenic activities of these compounds and their synthetic analogs. Zhang *et al.*, *Proc. Natl. Acad. Sci. USA* <u>89</u>: 2399-2403 (1992) and Posner *et al.*, *J. Med. Chem.* <u>17</u>: 170-176 (1994).

Although inducer activity has been found in many different families of edible plants, the amounts are highly variable, depending on family, genus, species, variety, or cultivar of the plant selection and on growth and harvesting conditions. Thus, there is a need in the art to identify particular edible plants and methods of growing and preparing them that yield high levels of Phase 2 enzyme-inducer activity for chemoprotection. There is also a need for methods of growing and preparing edible plants that produce a known spectrum of specific inducers of Phase 2 enzyme activity in order to increase the efficiency with which specific carcinogens, or classes of carcinogens, are targeted for inactivation. In addition, there is a need for methods of plant breeding and selection to increase the level of Phase 2 inducer activity and to manipulate the spectrum of inducers produced in particular cultivars.

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SUMMARY OF THE INVENTION

It is an object of the present invention to provide food products and food additives that are rich in cancer chemoprotective compounds.

Another object of the present invention is to provide food products which contain substantial quantities of Phase 2 enzyme-inducers and are essentially free of Phase 1 enzyme-inducers.

It is a further object of the present invention to provide food products which contain substantial quantities of Phase 2 enzyme-inducing potential and non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates.

These objects, and others, are achieved by providing cruciferous sprouts, with the exception of cabbage, cress, mustard and radish sprouts, harvested prior to the 2-leaf stage. The cruciferous sprouts include *Brassica oleracea* varieties *acephala*, *alboglabra*, *botrytis*, *costata*, *gemmifera*, *gongylodes*, *italica*, *medullosa*, *palmifolia*, *ramosa*, *sabauda*, *sabellica*, and *selensia*.

Another embodiment of the present invention provides cruciferous sprouts, with the exception of cabbage, cress, mustard and radish sprouts, harvested prior to the 2-leaf stage, wherein the sprouts are substantially free of Phase 1 enzyme-inducing potential.

Yet another embodiment of the present invention provides a non-toxic solvent extract of cruciferous sprouts, with the exception of cabbage, cress, mustard and radish sprouts, harvested prior to the 2-leaf stage. The non-toxic solvent extract can be a water extract. In addition, the water extract can comprise a cruciferous vegetable, such as a cruciferous vegetable of the genus *Raphanus*, comprising an active myrosinase enzyme.

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Another embodiment of the present invention provides a food product comprising cruciferous sprouts, with the exception of cabbage, cress, mustard and radish sprouts, harvested prior to the 2-leaf stage; extracts of the sprouts or cruciferous seeds; or any combination of the sprouts or extracts.

A further embodiment of the present invention provides a method of increasing the chemoprotective amount of Phase 2 enzymes in a mammal, comprising the step of administering an effective quantity of cruciferous sprouts, with the exception of cabbage, cress, mustard and radish sprouts, harvested prior to the 2-leaf stage.

Yet another embodiment of the present invention provides a method of increasing the chemoprotective amount of Phase 2 enzymes in a mammal, comprising the step of administering an effective quantity of a food product comprising cruciferous sprouts, with the exception of cabbage, cress, mustard and radish sprouts, harvested prior to the 2-leaf stage.

Another embodiment of the present invention provides cruciferous sprouts harvested prior to the 2-leaf stage, wherein the sprouts have at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days of growth from seeds that produce said sprouts and contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates. The cruciferous sprouts include *Brassica oleracea* varieties *acephala, alboglabra, botrytis, costata, gemmifera, gongylodes, italica, medullosa, palmifolia, ramosa, sabauda, sabellica,* and *selensia*.

A further embodiment of the present invention provides a food product comprising sprouts harvested prior to the 2-leaf stage, wherein the sprouts have at

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least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days from growth of seeds that produce the sprouts and contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates; extracts of the sprouts or cruciferous seeds; or any combination of the sprouts or extracts.

Yet another embodiment of the present invention provides cruciferous sprouts harvested prior to the 2-leaf stage, wherein the sprouts have at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days of growth from seeds that produce the sprouts and contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates and are substantially free of Phase 1 enzyme-inducing potential.

Another embodiment of the present invention provides a non-toxic solvent extract of cruciferous sprouts harvested prior to the 2-leaf stage, wherein the sprouts have at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days of growth from seeds that produce the sprouts and contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates. The non-toxic solvent extract can be a water extract. In addition, the water extract can comprise a cruciferous vegetable, such as a cruciferous vegetable of the genus *Raphanus*, comprising an active myrosinase enzyme.

Yet another embodiment of the present invention provides a method of increasing the chemoprotective amount of Phase 2 enzymes in a mammal, comprising the step of administering an effective quantity of cruciferous sprouts harvested prior to the 2-leaf stage, wherein the sprouts have at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when

measured after 3 days of growth from seeds that produce the sprouts and contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates.

Yet another embodiment of the present invention provides a method of increasing the chemoprotective amount of Phase 2 enzymes in a mammal, comprising the step of administering an effective quantity of a food product comprising sprouts harvested prior to the 2-leaf stage, wherein the sprouts have at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days of growth from seeds that produce the sprouts and contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates.

A further embodiment of the present invention provides a method of preparing a food product rich in glucosinolates, comprising germinating cruciferous seeds, with the exception of cabbage, cress, mustard and radish seeds, and harvesting sprouts prior to the 2-leaf stage to form a food product comprising a plurality of sprouts. The cruciferous sprouts include *Brassica oleracea* varieties *acephala*, *alboglabra*, *botrytis*, *costata*, *gemmifera*, *gongylodes*, *italica*, *medullosa*, *palmifolia*, *ramosa*, *sabauda*, *sabellica*, and *selensia* and contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates.

Yet another embodiment of the present invention provides a food product rich in glucosinolates made by germinating cruciferous seeds, with the exception of cabbage, cress, mustard and radish seeds, and harvesting sprouts prior to the 2-leaf stage to form a food product comprising a plurality of sprouts.

Yet another embodiment of the present invention provides a method of preparing a food product comprising

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extracting glucosinolates and isothiocyanates from cruciferous sprouts, with the exception of cabbage, cress, mustard and radish sprouts, harvested prior to the 2-leaf stage, with a non-toxic solvent and recovering the extracted glucosinolates and isothiocyanates. Myrosinase enzyme, or a vegetable, such as *Raphanus* species, containing the enzyme is mixed with the cruciferous sprouts, the extract, or both the sprouts and the extract.

An embodiment of the present invention provides a method of preparing a food product rich in glucosinolates, comprising germinating cruciferous seeds having at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days of growth from seeds that produce the sprouts and which contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates, and harvesting sprouts prior to the 2-leaf stage to form a food product comprising a plurality of sprouts. The seeds may be *Brassica oleracea*, including the varieties *acephala*, *alboglabra*, *botrytis*, *costata*, *gemmifera*, *gongylodes*, *italica*, *medullosa*, *palmifolia*, *ramosa*, *sabauda*, *sabellica*, and *selensia*.

Yet another embodiment of the present invention provides a food product rich in glucosinolates made by germinating cruciferous seeds having at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days of growth from seeds that produce the sprouts and which contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates, and either harvesting sprouts at the 2-leaf stage to form a food product comprising a plurality of sprouts. The nutritional product contains non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates.

A further embodiment of the present invention provides a method of preparing a food product comprising extracting glucosinolates and isothiocyanates with a solvent from cruciferous seeds, sprouts, plants or plant parts, wherein seeds that produce the sprouts, plants or plant parts producing sprouts having at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days of growth and wherein the seeds, sprouts, plants or plant parts have non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates, and recovering the extracted glucosinolates and isothiocyanates. The non-toxic extraction solvent can be water. Myrosinase enzyme, or a vegetable, such as *Raphanus* species, containing the enzyme is mixed with the cruciferous sprouts, seeds, plants, plant parts or extract, or any combination thereof.

A further embodiment of the present invention provides a method of reducing the level of carcinogens in mammals, comprising administering cruciferous sprouts, with the exception of cabbage, cress, mustard and radish sprouts.

Yet another embodiment of the present invention provides a method of reducing the level of carcinogens in mammals, comprising administering cruciferous sprouts having at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days of growth from seeds that produce the sprouts and non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates.

Another embodiment of the present invention provides a method of preparing a food product by introducing cruciferous seeds, having at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when

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measured after 3 days of growth from seeds that produce the sprouts and non-toxic levels of indole glucosinolates and goitrogenic hydroxybutenyl glucosinolates, into an edible ingredient.

A further embodiment of the present invention provides a method of extracting glucosinolates and isothiocyanates from plant tissue which comprises homogenizing the plant tissue in an excess of a mixture of dimethyl sulfoxide, acetonitrile, and dimethylformamide (DMF/ACN/DMSO) at a temperature that prevents myrosinase activity.

Another embodiment of the present invention provides cruciferous sprouts harvested prior to the 2-leaf stage, wherein the ratio of monofunctional to bifunctional inducers is at least 20 to 1.

Another object of the present invention is to provide a food product supplemented with a purified or partially purified glucosinolate.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the total inducing potential of organic solvent extracts of broccoli and daikon cultivars as a function of age.

Figure 2 shows the high resolution NMR spectra of isolated glucosinolates obtained from hot aqueous extracts of 3-day old Saga broccoli sprouts.

DETAILED DESCRIPTION

I. Definitions

In the description that follows, a number of terms are used extensively. The following definitions are provided to facilitate understanding of the invention.

A bifunctional inducer is a molecule which increases activities of both Phase 1 enzymes such as cytochromes P-450 and Phase 2 enzymes and requires the participation of Aryl hydrocarbon (*Ah*) receptor and its cognate Xenobiotic Response Element (*XRE*). Examples include flat planar aromatics such as polycyclic hydrocarbons, azo dyes or 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD).

A **chemoprotector** or **chemoprotectant** is a synthetic or naturally occurring chemical agent that reduces susceptibility in a mammal to the toxic and neoplastic effects of carcinogens.

A **food product** is any ingestible preparation containing the sprouts of the instant invention, or extracts or preparations made from these sprouts, which are capable of delivering Phase 2 inducers to the mammal ingesting the food product. The food product can be freshly prepared such as salads, drinks or sandwiches containing sprouts of the instant invention. Alternatively, the food product containing sprouts of the instant invention can be dried, cooked, boiled, lyophilized or baked. Breads, teas, soups, cereals, pills and tablets, are among the vast number of different food products contemplated.

Inducer activity or **Phase 2 enzyme-inducing activity** is a measure of the ability of a compound(s) to induce

Phase 2 enzyme activity. In the present invention, inducer activity is measured by means of the murine hepatoma cell bioassay of QR activity *in vitro*. Inducer activity is defined herein as QR inducing activity in Hepa 1c1c7 cells (murine hepatoma cells) incubated with extracts of sprouts, seeds or other plant parts untreated with myrosinase. Inducer activity is measured in Hepa 1c1c7 murine hepatoma cells grown in 96-well microtiter plates. Typically 10,000 Hepa 1c1c7 cells are introduced into each well. Hepatoma cells are grown for 24 hours and a plant extract containing microgram quantities of fresh plant tissue is serially diluted across the microtiter plates into fresh culture medium containing 0.15 ml αMEM culture medium amended with 10% Fetal Calf Serum (FCS) and streptomycin and penicillin. The cells are further incubated for 48 hours. QR activity (based on the formation of the blue-brown reduced tetrazolium dye) is measured with an optical microtiter plate scanner in cell lysates prepared in one plate, and related to its protein concentration. Quantitative information on specific activity of QR is obtained by computer analysis of the absorbances. One unit of inducer activity is the amount that when added to a single microtiter well doubles the QR activity. (*See* Prochaska and Santamaria, *Anal. Biochem.* 169: 328-336 (1988) and Prochaska et al., *Proc. Natl. Acad. Sci. USA* 89: 2394-2398 (1992)).

Inducer potential or Phase 2 enzyme-inducing potential is a measure of the combined amounts of inducer activity in plant tissue provided by isothiocyanates, plus glucosinolates that can be converted by myrosinase to isothiocyanates. Glucosinolates are not themselves inducers of mammalian Phase 2 enzymes, whereas isothiocyanates are inducers. Inducer potential therefore is defined herein as QR activity in murine 1c1c7 hepatoma cells incubated with myrosinase-treated extracts of the sprouts, seeds or other plant parts. In the present invention therefore inducer potential is measured by means of the murine hepatoma cell bioassay of

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QR activity *in vitro* as described above. Inducer potential is measured in Hepa 1c1c7 murine hepatoma cells grown in 96-well microtiter plates. Typically, 10,000 Hepa 1c1c7 cells are introduced into each well. Hepatoma cells are grown for 24 hours and a plant extract containing microgram quantities of fresh plant tissue is serially diluted across the microtiter plates into fresh culture medium containing 0.15 ml αMEM culture medium amended with 10% Fetal Calf Serum (FCS) and streptomycin and penicillin. Myrosinase (6 units/ml plant extract) is added to the plant extract. Myrosinase is purified by modification of the technique of Palmieri *et al.*, *Anal. Biochem.* 35: 320-324 (1982) from 7 day old Daikon sprouts grown on agar support containing no added nutrients. Following 234-fold purification, the myrosinase had a specific activity of 64 units/mg protein [unit = amount of enzyme required to hydrolyze 1 μmol sinigrin/min]. Plant extract is diluted 200-fold into the initial wells of the microtiter plate followed by 7 serial dilutions. The cells are further incubated for 48 hours. QR activity (based on the formation of the blue-brown reduced tetrazolium dye) is measured with an optical microtiter plate scanner in cell lysates prepared in one plate, and related to its protein concentration. Quantitative information on specific activity of QR is obtained by computer analysis of absorbances. One unit of inducer potential is the amount that when added to a single microtiter well doubles the QR activity. (*See* Prochaska and Santamaria, *Anal. Biochem.* 169: 328-336 (1988) and Prochaska *et al.*, *Proc. Natl. Acad. Sci. USA* 89: 2394-2398 (1992)).

A **monofunctional inducer** increases the activity of Phase 2 enzymes selectively without significantly altering Phase 1 enzyme activities. Monofunctional inducers do not depend on a functional *Ah* receptor but enhance transcription of Phase 2 enzymes by means of an Antioxidant Responsive Element (ARE).

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A cruciferous sprout is a plant or seedling that is at an early stage of development following seed germination. Cruciferous seeds are placed in an environment in which they germinate and grow. The cruciferous sprouts of the instant invention are harvested following seed germination through and including the 2-leaf stage. The cruciferous sprouts of instant invention have at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential at 3-days following incubation under conditions in which cruciferous seeds germinate and grow.

II. Description

A major mechanism of protection provided by fruits and vegetables in reducing the cancer incidence in humans depends on minor chemical components which, when delivered to mammalian cells, elevate levels of Phase 2 enzymes that detoxify carcinogens. It has now been discovered that the anticarcinogenic activity of certain edible plants can be increased. Plants such as *Brassica oleracea* variety *italica* (broccoli) are normally not harvested until they form heads. By growing these plants only to the seedling or sprout stage, that is between the onset of germination and the 2-leaf stage, the levels of inducers of enzymes that detoxify carcinogens and protect against cancer can be increased at least five-fold over those found in commercial stage vegetables of the same cultivars. Often increases of between 10 and 1000-fold have been observed.

Harvesting plants at an early seedling or sprout stage, or otherwise arresting their growth, leads to the greatest inducer potential and yields a food product of a type to which consumers are already accustomed. The Phase 2 enzyme-inducing potential of such sprouts may be as much as several hundred times higher than that observed in adult, market stage vegetables obtained from the same seeds. Thus it is possible that humans can consume the same quantities of inducer potential by

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eating relatively small quantities of sprouts, rather than large quantities of market-stage vegetables.

It has now been found that most of the inducer potential of crucifer plants is due to their content of isothiocyanates and their biogenic precursors, glucosinolates. Glucosinolates are converted to isothiocyanates by the enzyme myrosinase which is a thioglucosidase. Normally myrosinase and glucosinolates are separated in the cell and if the cell is damaged, with loss of compartmentalization, myrosinase comes into contact with glucosinolates, which are then converted to isothiocyanates.

In order to screen large numbers of edible plants and to evaluate the effects of environmental perturbation on Phase 2 enzyme-inducer potential in those vegetables, it was necessary to improve upon the previously described techniques for homogenization and extraction of those vegetables. Techniques initially described for the extraction of Phase 2 inducers from vegetables involved homogenization of the vegetables in cold water, lyophilization, extraction of the resultant powder with acetonitrile, filtration and evaporative concentration, Prochaska *et al.*, *Proc. Natl. Acad. Sci. USA* 89: 2394-2398 (1992).

Following identification of sulforaphane as the principal Phase 2 inducer from broccoli, comparative extractions were performed into hot 80% methanol, yielding similar inducer activity as the aforementioned acetonitrile extracts. When myrosinase was added to these hot methanol extracts in which glucosinolates are freely soluble, there was a dramatic enhancement of the Phase 2 inducer activity of these extracts (data summarized in Table 1). The deliberate conversion of these glucosinolates to isothiocyanates using exogenous myrosinase thus gave a better index of the inducers for Phase 2 enzymes of the vegetables tested. It was thus

clear that the majority of the potential Phase 2 inducers in crucifers was usually present in whole plants as the glucosinolate precursors of isothiocyanates.

The preponderance of glucosinolates and the rapidity with which, upon wounding of cruciferous plant tissue, glucosinolates are converted to isothiocyanates, led to the development of an improved extraction procedure. By manipulation of solvent mixtures and of the water activity of fresh vegetable/solvent homogenates, a procedure was developed that permits both glucosinolate and isothiocyanate quantification from the same, non-concentrated sample. In addition to being the rate-limiting step in an extraction protocol, evaporative concentration allows volatile inducers to escape detection. The improved procedure is both simple and efficient, requiring only that the plant sample be completely homogenized in solvent. Using this technique, the present inventors have thus been able to demonstrate dramatic increases in the recovery of inducer activity and inducer potential from cruciferous vegetables over previously described techniques.

If fresh-picked vegetables are promptly and gently harvested, directly into organic solvents comprising a mixture of DMF/ACN/DMSO and a temperature that prevents myrosinase activity, both glucosinolates and isothiocyanates are efficiently extracted into the organic solvent mixture. Preferably, the DMF, ACN and DMSO are mixed in equal volumes. However, the volumes of the three solvents in the mixture can be varied to optimize extraction of specific glucosinolates and isothiocyanates from any plant tissue. The temperature of the extraction mixture is preferably less than 0°C, and most preferably less than -50°C. The temperature of the extraction solvent must be kept above freezing. At the same time the enzyme myrosinase, which invariably accompanies these constituents in the plants and rapidly converts glucosinolates into isothiocyanates, is

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inactive. Such extracts typically contain high quantities of glucosinolates and negligible quantities of isothiocyanates. The *in planta* myrosinase activity varies between different plant species.

Glucosinolates are not themselves inducers of mammalian Phase 2 enzymes, whereas isothiocyanates are monofunctional inducers in the murine hepatoma cell bioassay of QR activity. The inducer potential, as distinct from inducer activity, of plant extracts can be measured by adding purified myrosinase, obtained from the same, or other plant sources, to the assay system.

Glucosinolates are converted at least partially to isothiocyanates in humans. If, however, it is desirable to accelerate this conversion, broccoli or other vegetable sprouts, high in glucosinolates, can be mixed with myrosinase. The mixture can be in water, or some other non-toxic solvent that does not inactivate myrosinase. The myrosinase can be from a partially purified or purified preparation. Alternatively, the myrosinase can be present in plant tissue, such as a small quantity of crucifer sprouts rich in myrosinase, including *Raphanus sativus* or daikon. Such a preparation can be used to produce a "soup" for ingestion that is high in isothiocyanates and low in glucosinolates. Inducer potential can be measured using a multiwell plate screen with murine hepatoma cells for *in vitro* measurement of QR specific activity as described above.

The ratio of monofunctional to bifunctional inducer activity of plant tissue is measured by bioassaying plant extracts, as described above, not only in wild-type Hepa 1c1c7 cells, but also, in mutants designated c1 and BP^rc1 that have either defective *Ah* receptors or defective cytochrome P_1-450 genes, respectively. Prochaska and Talalay, *Cancer Research* 48: 4776-4782 (1988).

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A harvested sprout according to the present invention can be incorporated immediately into food products such as fresh salads, sandwiches or drinks. Alternatively, the growth of the harvested sprout can be arrested by some active human intervention, for example by refrigeration, at a stage of growth prior to the 2-leaf stage, typically between 1 and 14 days after germination of seeds. Growth arrest can also be accomplished by removing a sprout from its substrate and/or water source. Freezing, drying, baking, cooking, lyophilizing and boiling are among the many treatments that can be used to arrest growth. These may also be useful for either preserving myrosinase activity in the sprout (*e.g.*, lyophilizing) or for inactivating myrosinase activity in the sprout (*e.g.*, boiling), as is desired in a particular application.

The harvested sprout can also be allowed to mature further, under different growing conditions, prior to incorporation into a food product. For example, the sprout can be harvested at a very young age of development, such as 1 to 2 days after seed imbibition. The sprout can then be allowed to mature under different growing conditions, such as increased or decreased light intensity, temperature or humidity; exposure to ultraviolet light or other stresses; or addition of exogenous nutrients or plant growth regulators (hormones). The sprout is then immediately incorporated into a food product, such as for fresh consumption in salads. Alternatively, the growth of the sprout is arrested and/or further treated by means of lyophilization, drying, extracting with water or other solvents, freezing, baking, cooking, or boiling, among others.

A sprout is suitable for human consumption if it does not have non-edible substrate such as soil attached or clinging to it. Typically the sprouts are grown on a non-nutritive solid support, such as agar, paper towel,

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blotting paper, Vermiculite, Perlite, etc., with water and light supplied. Thus, if a sprout is not grown in soil, but on a solid support, it does not need to be washed to remove non-edible soil. If a sprout is grown in a particulate solid support, such as soil, Vermiculite, or Perlite, washing may be required to achieve a sprout suitable for human consumption.

Sprouts can be grown in containers which are suitable for shipping and marketing. Typically such containers are plastic boxes or jars which contain a wetted pad at the bottom. The containers allow light to penetrate while providing a mechanically protective barrier. Numerous methods for the cultivation of sprouts are known, as exemplified by U.S. Patent Nos. 3,733,745, 3,643,376, 3,945,148, 4,130,964, 4,292,760 or 4,086,725. Food products containing the sprouts of the instant invention can be stored and shipped in diverse types of containers such as jars, bags and boxes, among many others.

Sprouts suitable as sources of cancer chemoprotectants are generally cruciferous sprouts, with the exception of cabbage (*Brassica oleracea capitata*), cress (*Lepidiumsativum*), mustard (*Sinapis alba* and *S. niger*) and radish (*Raphanus sativus*) sprouts. The selected sprouts are typically from the family *Cruciferae*, of the tribe *Brassiceae*, and of the subtribe *Brassicinae*. Preferably the sprouts are *Brassica oleracea* selected from the group of varieties consisting of *acephala* (kale, collards, wild cabbage, curly kale), medullosa (marrowstem kale), ramosa (thousand head kale), *alboglabra* (Chinese kale), *botrytis* (cauliflower, sprouting broccoli), *costata* (Portuguese kale), *gemmifera* (Brussels sprouts), *gongylodes* (kohlrabi), *italica* (broccoli), *palmifolia* (Jersey kale), *sabauda* (savoy cabbage), *sabellica* (collards), and *selensia* (borecole), among others.

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Particularly useful broccoli cultivars to be used in the claimed method are Saga, DeCicco, Everest, Emerald City, Packman, Corvet, Dandy Early, Emperor, Mariner, Green Comet, Green Valiant, Arcadia, Calabrese Caravel, Chancellor, Citation, Cruiser, Early Purple Sprouting Red Arrow, Eureka, Excelsior, Galleon, Ginga, Goliath, Green Duke, Greenbelt, Italian Sprouting, Late Purple Sprouting, Late Winter Sprouting White Star, Legend, Leprechaun, Marathon, Mariner, Minaret (Romanesco), Paragon, Patriot, Premium Crop, Rapine (Spring Raab), Rosalind, Salade (Fall Raab), Samurai, Shogun, Sprinter, Sultan, Taiko, and Trixie. However, many other broccoli cultivars are suitable.

Particularly useful cauliflower cultivars are Alverda, Amazing, Andes, Burgundy Queen, Candid Charm, Cashmere, Christmas White, Dominant, Elby, Extra Early Snowball, Fremont, Incline, Milkyway Minuteman, Rushmore, S-207, Serrano, Sierra Nevada, Siria, Snow Crown, Snow Flake, Snow Grace, Snowbred, Solide, Taipan, Violet Queen, White Baron, White Bishop, White Contessa, White Corona, White Dove, White Flash, White Fox, White Knight, White Light, White Queen, White Rock, White Sails, White Summer, White Top, Yukon. However, many other cauliflower cultivars are suitable.

Suitable sprouts will have at least 200,000 units per gram of fresh weight of Phase 2 enzyme-inducing potential following 3-days incubation of seeds under conditions in which the seeds germinate and grow. Preferably the sprouts will have at least 250,000 units of inducer potential per gram of fresh weight, or even 300,000 units, 350,000 units, 400,000 units, or 450,000 units. Some samples have been found to contain greater than 500,000 units per gram of fresh weight at 3-days of growth from seeds.

The level of inducing activity and inducing potential has been found to vary among crucifers and even among

cultivars. Most preferably, the sprouts are substantially free of indole glucosinolates and their breakdown products which have Phase 1 enzyme-inducing potential in mammalian cells, and substantially free of toxic levels of goitrogenic nitriles and glucosinolates such as hydroxybutenyl glucosinolates, which upon hydrolysis yield oxazolidonethiones which are goitrogenic. Mature Brussels sprouts and rapeseed are rich in these undesirable glucosinolates.

Non-toxic solvent extracts according to the invention are useful as healthful infusions or soups. Non-toxic or easily removable solvents useful for extraction according to the present invention include water, liquid carbon dioxide or ethanol, among others. The sprouts can be extracted with cold, warm, or preferably hot or boiling water which denature or inactivate myrosinase. The residue of the sprouts, post-extraction, may or may not be removed from the extract. The extraction procedure may be used to inactivate myrosinase present in the sprouts. This may contribute to the stability of the inducer potential. The extract can be ingested directly, or can be further treated. It can, for example, be evaporated to yield a dried extracted product. It can be cooled, frozen, or freeze-dried. It can be mixed with a crucifer vegetable which contains an active myrosinase enzyme. This will accomplish a rapid conversion of the glucosinolates to isothiocyanates, prior to ingestion. Suitable vegetables that contain active myrosinase are of the genus *Raphanus*, especially daikon, a type of radish.

Seeds, as well as sprouts have been found to be extremely rich in inducer potential. Thus it is within the scope of the invention to use crucifer seeds in food products. Suitable crucifer seeds may be ground into a flour or meal for use as a food or drink supplement. The flour or meal is incorporated into breads, other baked goods, or health drinks or shakes. Alternatively, the seeds may be extracted with a non-toxic solvent such as

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water, liquid carbon dioxide or ethanol to prepare soups, teas or other drinks and infusions. The seeds can also be incorporated into a food product without grinding. The seeds can be used in many different foods such as salads, granolas, breads and other baked goods, among others.

Food products of the instant invention may include sprouts, seeds or extracts of sprouts or seeds taken from one or more different crucifer genera, species, varieties, subvarieties or cultivars. It has been found that genetically distinct crucifers produce chemically distinct Phase 2 enzyme-inducers. Different Phase 2 enzyme-inducers detoxify chemically distinct carcinogens at different rates. Accordingly, food products composed of genetically distinct crucifer sprouts or seeds, or extracts or preparations made from these sprouts or seeds, will detoxify a broader range of carcinogens.

Glucosinolates and/or isothiocyanates can be purified from seed or plant extracts by methods well known in the art. *See* Fenwick *et al.*, *CRC Crit. Rez. Food Sci. Nutr.* 18: 123-201 (1983) and Zhang *et al.*, *Pro. Natl Acad. Sci. USA* 89: 2399-2403 (1992). Purified or partially purified glucosinolate(s) or isothiocyanate(s) can be added to food products as a supplement. The dose of glucosinolate and/or isothiocyanate added to the food product preferably is in the range of 1 μmol to 1,000 μmols. However, the dose of glucosinolate and/or isothiocyanate supplementing the food product can be higher.

The selection of plants having high Phase 2 enzyme-inducer potential in sprouts, seeds or other plant parts can be incorporated into *Cruciferae* breeding programs. In addition, these same breeding programs can include the identification and selection of cultivars that produce specific Phase 2 enzyme-inducers, or a particular spectrum of Phase 2 enzyme-inducers. Strategies for the crossing, selection and breeding of new cultivars of

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Cruciferae are well known to the skilled artisan in this field. *Brassica Crops and Wild Allies: Biology & Breeding*; S. Tsunoda et al. (eds), Japan Scientific Societies Press, Tokyo pp. 354 (1980). Progeny plants are screened for Phase 2 inducer activity or the chemical identity of specific Phase 2 enzyme-inducers produced at specific plant developmental stages. Plants carrying the trait of interest are identified and the characteristic intensified or combined with other important agronomic characteristics using breeding techniques well known in the art of plant breeding.

Example 1

COMPARISON OF CRUCIFEROUS SPROUT INDUCING POTENTIAL

Sprouts were prepared by first surface sterilizing seeds of different species from the cruciferae family with a 1 min treatment in 70% ethanol, followed by 15 min in 1.3% sodium hypochlorite containing approximately 0.001% Alconox detergent. Seeds were grown in sterile plastic containers at a density of approximately 8 seeds/cm^2 for from 1 to 9 days on a 0.7% agar support that did not contain added nutrients. The environment was carefully controlled with broad spectrum fluorescent lighting, humidity and temperature control. The seeds and sprouts were incubated under a daily cycle of 16 hours light at 25°C and 8 hours dark at 20°C.

Sprouts were harvested following 3-days of incubation and immediately plunged into 10 volumes of a mixture of equal volumes of DMF/ACN/DMSO at -50°C. This solvent mixture has a freezing point of approximately -33°C, but when admixed with 10% water, as found in plant material, the freezing point is depressed to below -64°C. The actual freezing point depression is even greater with plant material.

Homogenization was accomplished either by manually grinding the samples in a glass-on-glass homogenizer in

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the presence of a small amount of the total solvent used, then gradually adding more solvent or homogenizing the sample in 10 volumes of solvent using a Brinkman Polytron Homogenizer for 1 min at half-maximum power. The homogenate was then centrifuged to remove remaining particulates and stored at -20°C until assayed.

Inducer potential of plant extracts prepared as described above, was determined by the microtiter plate bioassay method as described in the Definitions section above.

Broccoli and cauliflower sprouts harvested and assayed at 3-days after incubation of seeds under growth conditions have Phase 2 enzyme-inducer potential greater than 200,000 units/g fresh weight. On the other hand, cabbage, radish, mustard and cress have Phase 2 enzyme-inducer potential of less than 200,000 units/g fresh weight when assayed at the same time point.

Example 2

VARIATION IN INDUCER POTENTIAL AMONG DIFFERENT BROCCOLI CULTIVARS

There is variation in inducer potential among different broccoli cultivars. In addition, most of the inducer potential in crucifers is present as precursor glucosinolates. The inducer activity and inducer potential of market stage broccoli heads was determined following DMF/ACN/DMSO extractions and assay of QR activity as described above.

Bioassay of homogenates of such market stage broccoli heads, with and without the addition of purified plant myrosinase, showed that the amount of QR activity found in the absence of myrosinase was less than 5% of that observed with added myrosinase. These observations confirmed previous suggestions (see Matile *et al.*,

Biochem. Physiol. Pflanzen 179: 5-12 (1984)) that uninjured plants contain almost no free isothiocyanates.

TABLE 1

Effect of Myrosinase on Inducer Activity of Market-Stage Broccoli Plant Heads

Broccoli cultivar	Units per gram (wet weight) vegetable	
	-myrosinase	+myrosinase
DeCicco	5,882	37,037
Calabrese Corvet	1,250	41,666
Everest	*	8,333
Dandy Early	*	20,000
Emperor	*	13,333
Saga	5,000	13,333
Emerald City	*	12,500

* Below limits of detection (833 units/g).

As can be observed in Table 1, most of the plant inducer potential is derived from glucosinolates following hydrolysis by myrosinase to form isothiocyanates. Hence, hydrolysis is required for biological activity.

Example 3

INDUCER POTENTIAL IS HIGHEST IN SEEDS AND DECREASES AS SPROUTS MATURE

Phase 2 enzyme-inducer potential is highest in seeds and decrease gradually during early growth of seedlings. Plants were prepared by first surface sterilizing seeds of *Brassica oleracea* variety *italica* cultivars Saga and DeCicco with a 1 min treatment in 70% ethanol, followed